Activation of α4β2*/α6β2* Nicotinic Receptors Alleviates Anxiety During Nicotine Withdrawal Without Upregulating Nicotinic Receptors

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2. Running Title Page.

Running Title: Anxiolytic effects and nAChR regulation of ABT-089

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Number of text pages: 34
Number of tables: 0
Number of figures: 5
Number of references: 51
Number of words in the Abstract: 219
Number of words in the Introduction: 750
Number of words in the Discussion: 1456

Nonstandard abbreviations: nAChR, nicotinic acetylcholine receptor; novelty-induced hypophagia, NIH

Recommended section assignment: Behavioral Pharmacology
3. Abstract.

While nicotine mediates its effects through several nicotinic acetylcholine receptor (nAChR) subtypes, it remains to be determined which nAChR subtypes directly mediate heightened anxiety during withdrawal. Relative success in abstinence has been found with the nAChR partial agonist Varenicline (Chantix; Pfizer), however treatment with this drug fails to alleviate anxiety in individuals during nicotine withdrawal. Therefore, it is hypothesized that success can be found by the repurposing of other nAChR partial agonists for cessation therapies that target anxiety. Interestingly, the selective partial agonists for α4β2, ABT-089, and α7, ABT-107, (AbbVie) have not been evaluated as possible therapeutics for nicotine cessation. Therefore we examined the effect of ABT-089 and ABT-107 on anxiety during withdrawal from nicotine in the novelty-induced hypophagia (NIH) paradigm. We found that acute ABT-089 and ABT-107 alleviate anxiety-like behavior during withdrawal from nicotine while chronic ABT-089 but not chronic ABT-107 reduces anxiety-like behavior during withdrawal. Following behavioral testing, brains were harvested and beta2-containing nAChRs were measured using [3H]Epibatidine. ABT-089 and ABT-107 do not upregulate nAChRs, which is in contrast to the upregulation of nAChRs observed following nicotine. Furthermore, ABT-089 is anxiogenic in nicotine naïve animals, suggesting that the effects on anxiety are specifically related to the nicotine-dependent state. Together, these studies identify additional nAChR partial agonists that may aid in the rational development of smoking cessation aids.
4. Introduction.

Cigarette smoking is the leading cause of death in the United States each year (Centers for Disease Control and Prevention (CDC), 2002). However, approximately 20% of the American population smokes despite the acknowledged health risks and socioeconomic costs (Centers for Disease Control and Prevention (CDC), 2011). In addition, maintenance of smoking cessation is at best modest, with 80% of smokers relapsing within the first year of quitting (Polosa and Benowitz, 2011). It is projected that if prevalence of use does not decrease from present rates, cigarette smoking and tobacco use will result in 10 million deaths per year by 2020 (Adhikari et al., 2009). Thus, identification of effective smoking cessation therapies is urgently needed.

Nicotine, which is the major addictive component in tobacco, plays a critical role in initial tobacco reinforcement and dependence (Le Foll and Goldberg, 2005). While many factors influence ongoing nicotine dependence, relapse to smoking is highly correlated with the severity of withdrawal symptoms present during abstinence (Ockene et al., 2000; Krall et al., 2002). These symptoms include difficulty concentrating, increased craving, depressed mood, and increased anxiety (Hughes, 1992; 2007). Varenicline (Chantix; Pfizer), a partial agonist at α4β2 nicotinic acetylcholine receptors (nAChRs) and a full agonist at α7 and α3β4 nAChRs is currently the best in class treatment for smoking cessation (Coe et al., 2005; Garrison and Dugan, 2009). However, while varenicline has been shown to improve both concentration and depressed mood and mitigate craving, recent studies in mice and human subjects have shown chronic treatment does not improve nicotine withdrawal-induced anxiety (Turner et al., 2013b; Cinciripini et al., 2013). This may be of special importance because
anxiety arising due to nicotine withdrawal has been correlated with relapse rates (Zhou et al., 2009).

Nicotine acts at multiple nAChR subtypes and understanding which subtypes contribute to the detrimental side effects experienced during withdrawal is critical for identification of novel and improved therapeutics. More specifically, it is important to examine how targeting of the cholinergic system can promote abstinence by reducing withdrawal-induced anxiety. The various subtypes of nAChRs play different roles in anxiety and nicotine withdrawal. For example, alterations in the activation of the α4 nAChR subunit result in heightened anxiety (Ross et al., 2000; Labarca et al., 2001). Furthermore, although α7 nAChRs have not been implicated directly in anxiety (Paylor et al., 1998; Vicens et al., 2011) they are necessary in the ventral tegmental area (VTA) for the expression of withdrawal from nicotine suggesting that targeting of the α7 subtype may also relieve nicotine withdrawal symptoms (Nomikos et al., 1999).

Chronic nicotine exposure produces a region specific upregulation of β2-containing nAChRs. This phenomenon is thought to contribute to nicotine addiction (Wonnacott, 1990; Marks et al., 1992; Buisson and Bertrand, 2002) and has been confirmed in several systems including cultured cells and rodent and human tissues (Marks et al., 1983; Schwartz and Kellar, 1985; Benwell et al., 1988; Peng et al., 1994; Breese et al., 1997; Perry et al., 1999; Staley et al., 2006). Chronic exposure to varenicline upregulates nAChRs, which parallels its anxiolytic effects during cessation from nicotine (Turner et al., 2011). However the more selective α4β2 nAChR compound, sazetidine-A, does not increase nAChRs despite behavioral anxiolytic
effects (Turner et al., 2010; Hussmann et al., 2012; Turner et al., 2013b). Therefore, the role of nAChR upregulation in mediating withdrawal-induced anxiety is as yet unclear.

ABT-089 (2-Methyl-3-(2(S)-pyrrolidinylmethoxy)pyridine; structure in Lin et al., 1997) is a selective partial agonist for \( \alpha_4\beta_2^* \) receptors with high selectivity for \( \alpha_4\alpha_5\beta_2 \) and activity at \( \alpha_6\beta_2^* \) receptors (Rueter et al., 2004; Marks et al., 2009). ABT-089 has been rigorously studied as a treatment for cognitive disorders, and has been used successfully in patients with Alzheimer’s disease (AD) and attention deficit disorder with few unintended side effects (Lin et al., 1997; Rueter et al., 2004). ABT-107 (5-(6-[(3R)-1-azabicyclo[2.2.2]oct-3-yloxy] pyridazin-3-yl)-1H-indole; structure in Bitner et al., 2010) is a selective agonist with high affinity at \( \alpha_7 \) nAChRs that has been characterized as a cognitive enhancer in animal models of AD and has low incidence of side effects at varying doses in patients (Bitner et al., 2010; Malysz et al., 2010; Othman et al., 2011). Both ABT-089 and ABT-107 enhance learning in naïve animals (Decker et al., 1997; Bitner et al., 2010). However, ABT-089 and ABT-107 have not been evaluated for their role in reducing anxiety following nicotine withdrawal or regulation of nAChRs. Therefore, we tested ABT-089 and ABT-107 and the selective targeting of distinct nAChR subtypes in order to identify which subtype may mediate anxiety during withdrawal from nicotine.
5. Materials and Methods.

Animals

Male 129SvJ;C57Bl/6J F1 hybrid mice (6-12 weeks of age, 20-30 g) were purchased from Taconic Farms (Hudson, NY), double-housed, and maintained on a 12-h light/dark cycle with food and water ad libitum in accordance with the University of Pennsylvania Animal Care and Use Committee. All experimental testing sessions were conducted between 9:00 a.m. and 1:00 p.m., with animals randomly assigned to treatment conditions and tested in counterbalanced order. For all studies, mice were acclimated to the behavioral testing facility for one hour prior to testing.

Drugs

Doses of nicotine tartrate (Sigma Aldrich, St. Louis, MO), ABT-089 and ABT-107 (synthesized by AbbVie, North Chicago, IL), are reported as free base weight. For acute studies, all drugs were prepared immediately before use in 0.9% saline and injected intraperitoneally. Dose response curves indicated that 1.2 mg/kg for ABT-089 and 0.03 mg/kg for ABT-107 (data not shown) do not alter locomotor activity in male 129SvJ;C57Bl/6J F1 hybrid mice.

For chronic treatment studies, nicotine (18 mg/kg/day), ABT-089 (0.769 mg/kg/day), and ABT-107 (0.32 mg/kg/day) were dissolved in 0.9% saline solution and administered for 14 days subcutaneously via osmotic minipump (model 2002; Alzet, Cupetino, CA). Mice were anesthetized with an isoflurane/oxygen mixture (1-3%), and osmotic minipumps were inserted subcutaneously using aseptic surgery techniques. Minipumps were placed parallel to the spine at shoulder level with the flow moderator
directed away from the surgical incision. The wound was closed with 7-mm stainless steel wound clips (Reflex; Cellpoint Scientific, Gaithersburg, MD).

Novelty-induced hypophagia

One week prior to the start of training, mice were housed in groups of two. Training days consisted of daily sessions in which mice were exposed to a highly palatable food (peanut butter chips; Nestle, Glendale, CA) presented in a clear plastic dish. During training and home cage testing sessions, a plastic insert (dividing the standard cage lengthwise) was used to separate mice in each cage. Mice were acclimated to the barriers one hour prior to presentation of the food. Food was placed in the cage for 15 minutes and latency to consume was measured (seconds). Training criterion was met once a latency under 20 seconds to approach and consume the food with <20% variability existed between mice.

EXPERIMENT 1: Acute administration

For acute ABT-089/ABT-107 drug treatment studies, mice were implanted with 14-day osmotic minipumps filled with nicotine or saline (For experimental design schematic see Figure 1A). On the last day of minipump viability, animals were tested in the home cage environment 10 minutes following an injection of saline. After the home test occurred, the nicotine minipump was removed in three-fourths of the nicotine treated animals and half of the saline treated animals. Twenty-four hours later, animals were acclimated for novel testing day and given an intraperitoneal injection ABT-089/ABT-107 or saline 10 minutes prior to testing in the novel environment. The novel
environment consisted of an empty standard cage with no bedding which was wiped with cleanser (1:10 pine sol dilution) to supply a novel odor, and placed in a white box with white light illumination (2150 lux). Latency to consume was recorded over 15 minutes. On both test days, the amount consumed (grams) of peanut butter chips was recorded.

EXPERIMENT 2: Chronic administration

For chronic drug treatment studies, mice were implanted with 14-day osmotic minipumps filled with nicotine or saline. After 14 days, nicotine minipumps were removed and replaced with a 14-day osmotic minipump containing either nicotine, ABT-089, or ABT-107. Following seven days of the 2nd treatment, osmotic minipumps were removed from half of the nicotine, ABT-089, and ABT-107 treatment groups to initiate spontaneous withdrawal. The remaining animals continued drug treatment for an additional seven days or underwent seven days of withdrawal from nicotine, ABT-089, or ABT-107. A home day was conducted and novel day testing occurred 24 hours later. Animals were sacrificed and brains were harvested for receptor binding experiments (For experimental design schematic see Figure 2a).

Receptor Binding

Mice used in the chronic ABT-089/ABT-107 experiment were sacrificed and used for the receptor binding experiment. Brain regions examined were constrained by a minimal tissue amount required for homogenate-binding assays. Tissues were harvested from animals immediately following behavioral testing. The samples were
homogenized in 50 mM Tris-HCl (Sigma Aldrich, St. Louis, MO) buffer, pH 7.4 at 24°C, and centrifuged twice at 35,000 x g for 10 minutes in fresh buffer. The membrane pellets were resuspended in fresh buffer and added to tubes containing a saturating concentration (2 nM) of [³H]epibatidine ([³H]EB; PerkinElmer, Boston, MA). [³H]EB binds is a high-affinity ligand for all heteromeric nACHRs with low nonspecific binding (Badio and Daly, 1994). [³H]EB was incubated with tissue in Tris buffer pH 7.4 for 2 hours at 24°C with [³H]EB. Bound receptors were separated from free ligand by vacuum filtration over GF/C glass fiber filters (Brandel, Gaithersburg, MD) that were pretreated with 0.5% polyethyleneimine (Sigma Aldrich, St. Louis, MO). The filters were then counted in a liquid scintillation counter. Nonspecific binding was determined in the presence of 300 µM nicotine, and specific binding was defined as the difference between total binding and nonspecific binding.

Statistical Analysis

All data are presented as mean± standard error of mean (SEM). For experiment 1, latency served as a dependent variable in two-way analysis of variance (ANOVA) followed post hoc by Bonferonni multiple comparisons test to detect differences. In experiment 2 (Figure 2) a repeated measures two-way ANOVA was used to determine significant differences between treatment groups with time (Home Day, Novel Day) as a repeated-measure (within) factor. A planned comparison (Bonferonni multiple comparison) was performed to test the hypothesis that chronic ABT-089 or ABT-107 administration during nicotine withdrawal show decreased latency to consume in a novel environment, comparing the Nic/ABT-089 and Nic/ABT-107 group with all other
groups. For receptor binding studies an ANOVA followed post hoc by Bonferroni multiple comparisons was used to detect differences between treatment groups. Statistical analyses were carried out using the GraphPad Prism 5.0 software package (GraphPad Software, San Diego, CA).
6. Results.

Acute ABT-089 and ABT-107 are anxiolytic during nicotine withdrawal in the NIH test

Withdrawal from chronic nicotine increases latency to consume in a novel environment and acute administration of ABT-089 and ABT-107 significantly reduces this latency (Figure 1b). There are significant differences between treatment groups on novel day (main effect of day, $F(1,35)=82.34$, $P < 0.0001$; main effect of treatment, $F(4,35)=9.736$, $P < 0.0001$; interaction, $F(4,35)=10.25$, $P < 0.0001$). Bonferroni post hoc analysis indicated that nicotine treated mice had significantly lower latency to consume ($P < 0.05$) while mice experiencing 24h withdrawal from nicotine had significantly greater latency to consume ($P < 0.001$) when compared to saline treated controls. An acute administration of ABT-107 significantly reduced latency to consume the food in the novel environment during 24h withdrawal from nicotine ($P < 0.01$) when compared to saline controls. Administration of ABT-107 and ABT-089 significantly reduced latency to consume at 24h withdrawal when compared to mice undergoing 24h withdrawal from nicotine ($P < 0.0001$). Similarly, animals maintained on nicotine showed a reduced latency to consume in the novel environment when compared to animals experiencing 24h withdrawal from nicotine ($P < 0.0001$). There was no change in amount consumed between home day and novel test day (data not shown).

Chronic ABT-089 is anxiolytic during nicotine withdrawal in the NIH test

To better model a therapeutic administration paradigm, we tested the impact of ABT-089 and ABT-107 on withdrawal induced anxiety following a chronic exposure of the drug. A schematic of the chronic administration paradigm is shown in Figure 2a. Data in Figure 2b demonstrate that 14 day administration of ABT-089 and ABT-107
during nicotine withdrawal reduced latency to consume compared to saline controls, although not significantly (main effect of day, $F(1,24)=18.22, P = 0.0003$). Planned comparison of Nic/ABT-089 and Nic/ABT-107 to other treatment groups revealed that when compared to nicotine withdrawal (7d), ABT-089 significantly reduced latency to consume ($P < 0.05$), while ABT-107 did not. Animals in which minipumps were removed to induce spontaneous withdrawal from ABT-089 or ABT-107 did not show reduced latency to consume in the novel environment as compared to saline controls or nicotine withdrawal.

**ABT-089 does not upregulate nAChRs during nicotine withdrawal**

As previously demonstrated, nicotine upregulates nAChRs and receptors return to basal levels as early as 24h into withdrawal from nicotine (Figure 3 and Turner et al., 2011). Chronic nicotine increases nAChRs in the hippocampus, cortex, striatum, and thalamus (Figure 3; main effect of treatment, Hippocampus: $(F(4,27)=8.462; P = 0.0001)$, Cortex: $(F(4,29)=15.91; P < 0.0001)$, Striatum: $(F(4,29)=8.017; P = 0.0002)$, Thalamus: $(F(4,28)=6.774; P = 0.0006)$. However, following seven days of withdrawal from nicotine (WD), nAChRs are no longer upregulated when compared to saline control animals. ABT-089 (Nic/ABT-089) and ABT-107 (Nic/ABT-107) administration over during withdrawal from nicotine does not maintain upregulated nAChRs when compared to saline control animals.

**ABT-089 alone does not upregulate nAChRs**

To determine if chronic administration of ABT-089 alone can upregulate nAChRs, brain regions of interest were harvested from animals exposed to ABT-089 for 14 days. Chronic administration of ABT-089 in nicotine naïve mice did not significantly upregulate
nAChRs in the hippocampus, cortex, striatum, or thalamus of treated animals compared to saline controls. Hippocampus: \((F(2,21)=0.3893; P = 0.6820)\). Cortex: \((F(2,19)=0.3749; P = 0.6923)\). Striatum: \((F(2,21)=0.9581; P = 0.3998)\). Thalamus: \((F(2,20)=2.985; P = 0.0734)\). Additionally, there is no up- or downregulation of receptors following 24h withdrawal from ABT-089 in brain regions chosen for analysis (Figure 4).

ABT-089 alone is anxiogenic in naïve animals

Since ABT-089 chronically blocked nicotine-induced anxiety in nicotine dependent mice, we tested the effects of chronic ABT-089 in nicotine naïve mice. An effect of day was revealed using two-way ANOVA \((F(1,21) = 61.06; P < 0.0001)\) however there was no effect of treatment on latency to consume (Figure 5). While, latency to consume in the novel environment appears greater in animals treated with chronic ABT-089 as well as in animals undergoing 24h withdrawal from chronic ABT-089 at the time of testing, this increase in latency is non-significant.
7. Discussion.

Acute administrations of ABT-089 and ABT-107 alleviate anxiety during nicotine withdrawal however only ABT-089 is effective in alleviating anxiety following chronic administration. Additionally, ABT-089 mediates its effects without upregulating nAChRs, a hallmark of sustained nAChR activation with nicotine. Therefore, ABT-089 may be an effective compound in treating individuals with heightened anxiety during nicotine abstinence through a different cellular mechanism than other cessation therapies.

While administration of varenicline during smoking cessation results in the improvement of both positive affect and cognitive function (Patterson et al., 2009), efficacy in alleviating anxiety during nicotine withdrawal is more complicated (Cinciripini et al., 2013). Specifically, acute but not chronic varenicline reduces anxiety during withdrawal. For example, in naïve animals, acute and chronic varenicline administration is anxiolytic in the NIH test (Turner et al., 2010). However, in nicotine-experienced animals, acute administration of varenicline during nicotine withdrawal fails to alleviate anxiety-like behavior (Turner et al., 2013b), suggesting a differential effect of varenicline based on the drug experienced state of the subject. Additionally, a recent study in smokers identified anxiety as one of the symptom domains in which varenicline treatment was ineffective (Cinciprini et al., 2013). Therefore this lack of anxiolytic activity during nicotine withdrawal may underlie the low success rate of varenicline in a subset of smokers (Garrison and Dugan, 2009; Moore et al., 2010). Because varenicline acts at a number of different nAChR subtypes, identifying those subtypes that underlie the beneficial effects of the drug while avoiding subtypes responsible for negative side effects is necessary. Additionally, using a more selective ligand to produce desirable
effects during nicotine withdrawal could provide tailored therapies to suit the individual needs of quitters.

Nicotine exerts its biological effects through activation of central nAChRs that exist as subtypes determined by α and β subunit compositions (Gotti and Clementi, 2004). The heteromeric α4β2* subtype and the homomeric α7 are the most prevalent receptor subtypes in the central nervous system (Court et al., 2000). Varenicline, the best in class medication for smoking cessation, acts at both of these subtypes (Coe et al., 2005). ABT-089 is selective for the α4β2* and α6β2* (Marks et al., 2009) subtypes that have been implicated in anxiety (Ross et al., 2000; Labarca et al., 2001). ABT-107 is selective for the α7 subtype (Malysz et al., 2010) that has been implicated in generalized nicotine withdrawal syndrome (Nomikos et al., 1999). Therefore, we used these highly-selective nicotinic compounds to determine the contribution of specific subtypes during nicotine withdrawal on anxiety using the NIH paradigm and further examined the effects of these drugs on receptor regulation with [3H]EB binding assay. The NIH paradigm is a sensitive measure for potential anxiolytic drugs and anxiogenic effects of withdrawal on both acute and extended drug administration paradigms (Dulawa et al., 2004; Dulawa and Hen, 2005). The NIH test is sensitive to the anxiolytic effect of chronic nicotine (Turner et al., 2010; Turner et al., 2013b) and the anxiogenic effect of 24h withdrawal from nicotine (as shown in Figure 1). Acute administration of ABT-089 and ABT-107 during 24h withdrawal from chronic nicotine treatment reduces anxiety-like behavior in animals (Figure 1b). However, chronic administration of ABT-089, but not ABT-107, during nicotine withdrawal showed a significant decrease in
latency to consume compared to animals undergoing seven day WD from nicotine (Figure 2b).

Nicotine withdrawal typically causes an increased latency to consume food in a novel environment above that of saline treated animals (Figure 1b and Turner et al., 2010; 2013a; 2013b). However, these observations are generally evident during the first 24 to 72 hours of nicotine withdrawal. In Figure 2b the long-term effects of nicotine withdrawal are observed and data demonstrate that increased anxiety occurs compared to chronic nicotine administration during nicotine withdrawal that persists beyond a 72 hour timepoint. Thus, chronic exposure of ABT-089 throughout the withdrawal period when individuals may be particularly vulnerable to withdrawal-induced anxiety is an important aspect of our study design. In humans, while most reports of anxiety occur during the first 24 hours, a return to baseline can take up to four weeks (Hughes, 1992). Therefore, the efficacy of ABT-089 to reduce anxiety within an extended time period suggests it would be an effective treatment for the alleviation of both the initial anxiety experienced during withdrawal (Figure 1) as well as anxiety over the course of abstinence. However, it should be noted that the anxiolytic effect of ABT-089 is only evident when drug is on board as latency to consume in a novel environment is increased 7 days after withdrawal from ABT-089 (Figure 2b).

Previous studies suggest that the upregulated pool of nAChRs arising from chronic exposure to nicotine may drive elements of the nicotine withdrawal syndrome (Turner et al., 2011; Gould et al., 2012) and this protracted upregulation of nAChRs has been correlated with reduced ability to maintain abstinence in the clinical population (Staley et al., 2006). Therefore, to determine if ABT-089 reduced anxiety during nicotine
withdrawal in the NIH via maintenance of upregulated nAChRs, we quantified [³H]EB binding density following chronic ABT-089 administration during nicotine withdrawal. Chronic ABT-089 does not upregulate heteromeric nAChRs during nicotine withdrawal (Figure 3). However, it is effective in alleviating anxiety during withdrawal (Figure 2b). Likewise, chronic ABT-107 does not upregulate heteromeric nAChRs (Figure 3). This outcome was expected as α7 nAChRs do not upregulate to the same degree as heteromeric nAChRs following chronic nicotine treatment (Mugnaini et al., 2002). These findings suggest that ABT-089 functionally reduces anxiety during nicotine withdrawal without maintaining upregulation of heteromeric nAChRs. Therefore, in contrast to varenicline, ABT-089 allows nAChRs to down-regulate back to saline levels while providing an anxiolytic effect during withdrawal from nicotine.

Due to the efficacy of ABT-089 in alleviating nicotine withdrawal-induced anxiety we sought to determine if chronic ABT-089 alone could reduce anxiety in the NIH, thereby broadening the clinical applications of this compound. In addition, we evaluated whether abstinence from chronic ABT-089 produces a withdrawal state that may impact anxiety-like behavior. We found that administration of ABT-089 for two weeks in drug-naïve animals did not decrease anxiety in the NIH. In addition, 24h withdrawal from ABT-089 did not significantly increase latency compared to saline treated animals (Figure 5). While a previous study showed ABT-089 decreased anxiety, this was following acute rather than chronic administration (Lin et al., 1997). In addition, unlike the decrease in latency observed with chronic nicotine administration (Figure 1b), ABT-089 administration fails to produce an anxiolytic effect. The vastly different behavioral effects of ABT-089 compared to nicotine suggest that the partial agonist activity
produces different biochemical effects compared to the promiscuous activity of nicotine. Additionally, our findings from [$^3$H]EB binding assays demonstrate that chronic ABT-089 administration in drug-naïve animals does not upregulate nAChRs (Figure 4), similar to its effects in nicotine dependent animals.

Our findings suggest that partial activation of $\alpha_4\beta_2^*/\alpha_6^*$ and $\alpha_7$ nAChRs may have an effect on the initial anxiety experienced during nicotine withdrawal and sustained activation of $\alpha_4\beta_2^*/\alpha_6^*$ nAChRs through chronic exposure to ABT-089 during nicotine withdrawal alleviates anxiety. This finding supports previous research implicating $\alpha_4$ in anxiety (Ross et al., 2000; Labarca et al., 2001). Additionally, $\beta_2$ knockout mice, and therefore bereft of $\alpha_4\beta_2^*$ heterodimers, have reduced anxiety during withdrawal from chronic nicotine and following mecamylamine precipitated withdrawal from nicotine (Jackson et al., 2008). Finally, a subset of $\alpha_6$-containing nAChRs ($\alpha_6\beta_2\beta_3^*$) regulate sustained release on dopamine nerve terminals and therefore may provide the efficacy through which both acute and chronic ABT-089 alleviate withdrawal symptoms (Marks et al., 2009). To date, $\alpha_7$ nAChRs have not been implicated in anxiety during nicotine withdrawal (Vicens et al., 2011) and they do not appear to be necessary for somatic signs of withdrawal (Markou and Paterson, 2001). However, as acute ABT-107 reduced latency to consume during nicotine withdrawal, the targeting of $\alpha_7$ nAChRs may relieve withdrawal symptoms by activating nAChRs in the absence of nicotine.

Finally, findings from this study suggest a complex association between nAChR regulation and behavior. This study is the first to demonstrate that the selective partial nAChR agonist ABT-089 reduces anxiety during nicotine withdrawal without maintaining
the upregulated pool of heteromeric nAChRs. However, while the ligand binding results are similar in drug-naïve and drug-experienced animals exposed to chronic administration of ABT-089, their behavioral profiles are strikingly different. These findings highlight the importance of drug screening in nicotine-experienced animals. Interestingly, ABT-107, a selective partial agonist at α7 nAChRs is capable of alleviating anxiety at 24h withdrawal from nicotine, but not following extended administration, suggesting the role of several types of nAChRs in the initial withdrawal symptoms following nicotine cessation that may differ when withdrawal is extended. Therefore, the temporal specificity of the roles of discrete nAChR subtypes following the onset of nicotine withdrawal needs to be further studied to better identify improved therapeutics for nicotine cessation.
8. Acknowledgements.

We thank AbbVie for generously providing ABT-089 and ABT-107 and Dr. Lynne Rueter (Associate Director II, Neuroscience Discovery; AbbVie) for helpful discussions. We thank Ms. Emily Fernandez for experimental assistance.

*Participated in research design:* Yohn, Turner, and Blendy.

*Conducted experiments:* Yohn.

*Performed data analysis:* Yohn and Turner.

*Wrote or contributed to the writing of the manuscript:* Yohn, Turner, and Blendy.
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11. Footnotes.

a) This work was supported by the National Institute of Health/National Institute for Drug Abuse/National Cancer Institute [Grants P50 CA143187, K99 DA032681, T32 DA28874].

b) This work was presented at the Society for Research on Nicotine and Tobacco 19th Annual International Conference (2013), P054-99.

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12. Figure Legends.

Figure 1. The effect of acute ABT-089 and ABT-107 on the behavior of mice during nicotine withdrawal in the NIH. A. Mice were implanted with osmotic minipumps containing nicotine or saline. After two weeks of treatment minipumps were removed from half of the nicotine animals to induce spontaneous nicotine withdrawal. ABT-089 and ABT-107 were administered intraperitoneally 10m prior to NIH testing. B. Latency to consume in the novel environment was measured over 15m and is reported as mean latency ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001 compared with saline on Novel Day. †P < 0.0001 compared to 24h WD on Novel Day. (n = 8-12).

Figure 2. The effect of chronic ABT-089 and ABT-107 on the behavior of mice during nicotine withdrawal in the NIH. Data are mean (± SEM) of treatment groups composed of five to nine mice per group. A. Mice were implanted with osmotic minipumps containing nicotine or saline. After two weeks of treatment minipumps were removed from half of the nicotine animals and replaced with a minipump containing ABT-089 or ABT-107. One week following minipump switch, minipumps were removed from half of the nicotine, ABT-089, and ABT-107 treatment groups to induce spontaneous withdrawal. B. Latency to consume in the novel environment two weeks following minipump switch and one week following removal of the second minipump animals were tested in the NIH and latency to consume was measured. Latency to consume in the novel environment two weeks following minipump exchange and 7d withdrawal from nicotine is reported as mean latency ± SEM. †P < 0.05 compared with nicotine withdrawal on Novel Day. (n = 4 – 9).
Figure 3. Nicotinic receptor regulation following nicotine withdrawal in the presence or absence of ABT-089 or ABT-107. Homogenate-binding experiments with a saturating concentration of \([^{3}\text{H}]\text{epibatidine}\) ([\(^{3}\text{H}]\text{EB}) were performed on hippocampal, cortical, striatal, and thalamic tissues from animals treated with chronically with nicotine for two weeks then chronically with ABT-089 or ABT-107 for two weeks during withdrawal from nicotine. Additional treatment groups include animals treated with chronic saline (four weeks), chronic nicotine (four weeks) and animals treated with nicotine for three weeks and then withdrawn from nicotine for 7d (via nicotine minipump removal and spontaneous withdrawal). Data are mean (± SEM) of treatment groups. *\(P < 0.01\), **\(P < 0.001\), ***\(P < 0.0001\) compared with saline. (n = 5 – 10).

Figure 4. Nicotinic receptor regulation following chronic treatment with ABT-089. Homogenate-binding experiments with a saturating concentration of \([^{3}\text{H}]\text{Epibatidine}\) ([\(^{3}\text{H}]\text{EB}) were performed on hippocampal, cortical, striatal, and thalamic tissues from animals treated chronically with ABT-089 or saline for two weeks. Minipumps were removed from half of the ABT-089 animals to induce spontaneous withdrawal for 24h. Data are mean (± SEM) of treatment groups. (n = 7 – 8).

Figure 5. The effect of chronic ABT-089 and withdrawal from ABT-089 on the behavior of mice in the NIH. Data are mean (± SEM) of treatment groups composed of eight mice per group. Mice were implanted with osmotic minipumps containing ABT-089 or saline. After two weeks of treatment minipumps were removed from half of the ABT-089
animals to induce spontaneous nicotine withdrawal. Twenty-four hours later latency to consume in a novel environment over 15m was measured in all treatment groups. Data is presented as mean latency (± SEM) of treatment groups. (n=8).
Figure 1

A.

Day 0  14  15
Saline
Nicotine
Nicotine
Injection and Behavior Testing

B.

Latency to feed (s)

Saline
Nicotine
24h WD
WD/ABT-089
WD/ABT-107

Home Day
Novel Day

0  100  200  300  400  500
Figure 2

A.

Saline
Nicotine
Nicotine
Nicotine ABT-089/ABT-107
Nicotine ABT-089/ABT-107

Behavior Testing

B.

Latency to feed (s)

Sal/Sal
Nic/Nic
Nic WD (7d)
Nic/ABT-089
Nic/ABT-107
Nic/ABT-089 WD (7d)
Nic/ABT-107 WD (7d)

Home Day
Novel Day
Figure 3

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JPET Fast Forward. Published on March 13, 2014 as DOI: 10.1124/jpet.113.211706
Figure 4

[Bar graph showing fmol/mg tissue levels for different brain regions: Hippocampus, Cortex, Striatum, and Thalamus, comparing Saline and ABT-089 WD conditions.]
Figure 5